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Interaction of p190RhoGAP with C-terminal Domain of p120-catenin Modulates Endothelial Cytoskeleton and Permeability*

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Background: p120-catenin protein interactions regulate vascular permeability.
Results: We identified p190RhoGAP-binding domain of p120-catenin and evaluated its functional significance.
Conclusion: Binding of p190RhoGAP occurs at the amino acid 820–843 domain of p120-catenin and promotes activation of Rac and down-regulation of Rho signaling, leading to increased endothelial barrier.
Significance: These data demonstrate functional significance of uncoupling the p120-catenin-p190RhoGAP interaction in the context of agonist-induced endothelial permeability.

p120-catenin is a multidomain intracellular protein, which mediates a number of cellular functions, including stabilization of cell-cell transmembrane cadherin complexes as well as regulation of actin dynamics associated with barrier function, lamellipodia formation, and cell migration via modulation of the activities of small GTPAses. One mechanism involves p120 catenin interaction with Rho GTPase activating protein (p190RhoGAP), leading to p190RhoGAP recruitment to cell periphery and local inhibition of Rho activity. In this study, we have identified a stretch of 23 amino acids within the C-terminal domain of p120 catenin as the minimal sequence responsible for the recruitment of p190RhoGAP (herein referred to as CRAD; catenin-RhoGAP association domain). Expression of the p120-catenin truncated mutant lacking the CRAD in endothelial cells attenuated effects of barrier protective oxidized phospholipid, OxPAPC. This effect was accompanied by inhibition of membrane translocation of p190RhoGAP, increased Rho signaling, as well as suppressed activation of Rac1 and its cytoskeletal effectors PAK1 (p21-activated kinase 1) and cortactin. Expression of p120 catenin-truncated mutant lacking CRAD also delayed the recovery process after thrombin-induced endothelial barrier disruption. Concomitantly, RhoA activation and downstream signaling were sustained for a longer period of time, whereas Rac signaling was inhibited. These data demonstrate a critical role for p120-catenin (amino acids 820–843) domain in the p120-catenin·p190RhoGAP signaling complex assembly, membrane targeting, and stimulation of p190RhoGAP activity toward inhibition of the Rho pathway and reciprocal up-regulation of Rac signaling critical for endothelial barrier regulation.

p120-catenin is a multidomain intracellular protein containing N-terminal regulatory domain, a central domain with 10 Armadillo repeats and a less well functionally defined C-terminal tail (1–4). One of the major functional roles of p120-catenin in mammalian cells is stabilizing cell-cell transmembrane cadherin molecules at the cell membrane by modulating their trafficking and degradation either through direct binding to the cadherin cytoplasmic tail or indirectly through regulating the trafficking machinery (1, 4–8). By virtue of its main localization in adherens junctions, p120-catenin plays an essential role in the maintenance of cell-cell adhesion and in the control of intercellular permeability of epithelial as well as endothelial cell monolayers *in vitro* and *in vivo* (9–12). In addition, a number of recent studies have shown that it plays an important role in vascular development, inflammation, and tumor progression and metastasis (13–17).

One of the many important roles of p120-catenin consists of its regulatory activity on actin dynamics associated with barrier function, lamellipodia formation, and cell migration through modulation of the activities of small GTPAses RhoA, Rac, and Cdc42 (17–22). Although p120-catenin-mediated inhibition of RhoA activity was shown to occur via a direct binding of RhoA to a region within the N-terminal regulatory domain (23), other studies suggest that it may also do so indirectly through recruitment and binding of the Rho family exchange factor Vav2 (24) or through functional interaction with Rho GTPase activating protein (p190RhoGAP) (25). Consistent with the latter, we have recently shown that p190RhoGAP plays a key role in integrating the opposed activities of Rac and Rho and the actin remodeling events involved in endothelial barrier enhancement by oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC)[3] (26). OxPAPC treatment of human pul-

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[3] The abbreviations used are: OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; HPAEC, human pulmonary artery endothelial cell(s); MLC, myosin light chain; MYPT, phospho-Thr[850] myosin-associated phosphatase; TER, transendothelial electrical resistance; GAP, GTPase activating protein; VE-cadherin, vascular endothelial cadherin; EC, endothelial cells; PAK1, p21-activated kinase 1.

TABLE 1
List of forward and reverse primers used to generate stop codons at the indicated positions within the C-terminal tail of p120 catenin

Primer sequence (5′→3′)	Orientation	Mutation	Mutant
GCAGCAGCTCTTGTCCTGTAGACAATCTGGGGCTATAAGG	Forward	Stop codon at aa 821[a]	GST-p120 1A (1–820)
CCTTATAGCCCCAGATTGTCTACAGGACAAGAGCTGCTGC	Reverse		
GGATGGAAAAATCAGACTTCTAGGTGAATCTAAACAATGC	Forward	Stop codon at aa 844	GST-p120 1A (1–843)
GCATTGTTTAGATTCACCTAGAAGTCTGATTTCTTCCATCC	Reverse		
CATATGATGATAGCACTCTCTGACTCATTGACCGGAATC	Forward	Stop codon at aa 865	GST-p120 1A (1–864)
GATTCCGGTCAATGAGTCAGAGAGTGCTATCATCATATG	Reverse		
CCAATGAGCAATATGGGGTAAAACACAAAATCATTAGATAAC	Forward	Stop codon at aa 889	GST-p120 1A (1–888)
GTTATCTAATGATTTTGTGTTTTACCCATATTGCTCATTGG	Reverse		
GAGAGAGGAGACCACAACTGAACACTGGACCGATCTGGGG	Forward	Stop codon at aa 909	GST-p120 1A (1–908)
CCCCAGATCGGTCCAGTGTTCAGTTGTGGTCTCCTCTCTC	Reverse		

[a] aa, amino acid.

monary artery endothelial cells led to tyrosine phosphorylation and recruitment of p190RhoGAP to the plasma membrane in a Rac-dependent manner where it then formed a complex with adherens junction protein p120 catenin (26). In this study, we have sought to identify the minimal sequence responsible for the interaction between p120-catenin and p190RhoGAP using a series of deletion and C-terminal truncation mutants and to characterize the functional significance of p120-catenin-p190RhoGAP molecular uncoupling. Tagged constructs were exogenously expressed in human embryonic kidney cells and analyzed by pulldown assays followed by Western blotting. The effects of p120-catenin mutants on Rac- and Rho-dependent signaling, cytoskeletal remodeling, and agonist-induced permeability changes were investigated upon transient transfection in primary culture of human pulmonary artery endothelial cells (HPAEC).

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Unless specified otherwise, biochemical reagents were obtained from Sigma. Reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Antibodies to diphospho-Ser[19]/Thr[18] myosin light chain (MLC), phospho-Ser[199]-PAK1 and phospho-Ser[423]-PAK1 were from Cell Signaling, Inc. (Beverly, MA). Antibodies to phospho-Thr[850] myosin-associated phosphatase (MYPT) and phospho-Tyr[421]-cortactin were from Millipore (Billerica, MA); antibodies to p120-catenin and p190RhoGAP were from BD Transduction Laboratories (San Diego, CA). Primary antibodies to GST tag were from Santa Cruz (Santa Cruz, CA), antibody to His tag from QED Bioscience (San Diego, CA), and fluorescently labeled (Alexa Fluor 488 and 555) secondary antibodies were from Invitrogen.

HEK293 T cells were cultured in DMEM/high glucose (Invitrogen) supplemented with 10% FBS (Mediatech) and maintained in a humidified tissue culture incubator at 37 °C and 5% CO_2. HEK 293 transfections were carried out using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were used 48 h after transfection. HPAEC were obtained from Lonza (Allendale, NJ), maintained in a complete culture medium according to the manufacturer's instructions, and used for experiments at passages 5–7. Transient transfections of HPAEC were carried out using PolyJet reagent from Signagen Laboratories (Rockville, MD) as recommended by the manufacturer. Cells were used 24 h after transfection.

Constructs—The original p120-catenin and p190RhoGAP constructs described previously (6, 22, 27, 28) were subcloned into the Gateway entry vector (pENTR/D-TOPO) then transferred by recombination reactions using LR clonase to the destination vectors pDEST26 (N-terminal histidine fusion) and pDEST27 (N-terminal GST fusion) (Invitrogen). All constructs were verified by sequencing prior to use.

PCR-based Mutagenesis—Stop codons were introduced at different positions within the C-terminal tail of p120-catenin (Table 1) using a PCR-based mutagenesis approach. Briefly, full-length p120 catenin construct was amplified by PCR in the presence of a selected pair of primers containing the desired mutation (Table 1). The PCR reaction mixture was then treated with DpnI restriction enzyme to eliminate the original methylated template plasmid DNA before retransforming into *Escherichia coli* bacteria. All mutations were confirmed by sequencing prior to use.

GST/His Pulldown—48 h after transfection, cells were washed in cold PBS and lysed on ice with cold TBS-Nonidet P-40 lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science). Clarified lysates were then incubated with either glutathione magnetic beads (Thermo Scientific) or His-Tag Dynabeads (Invitrogen) overnight at 4 °C and washed three to four times with TBS-Nonidet P-40 lysis buffer, and the complexes were analyzed by Western blotting using the appropriate antibodies. The fluorescent signals were acquired and analyzed using Pharos FX Plus molecular imager (Bio-Rad) as recommended by the manufacturer.

Rac and Rho Activation Assay—Rac and Rho activation was assessed using the GTP-bound GTPase pulldown assays as described previously (29, 30). Briefly, after the incubation with agonist, cell lysates were collected, and GTP-bound Rac was captured using pulldown assay with PAK1 binding domain (PBD)-agarose, whereas GTP-bound Rho was captured using pulldown assay with Rhotekin-agarose. The levels of activated Rac1 and Rho as well as total Rac1 and Rho content were evaluated by Western blot analysis and quantified by scanning densitometry of the autoradiography films. The levels of activated GTPases were normalized to total Rac or Rho content in cell lysates.

Measurements of Transendothelial Electrical Resistance (TER)—TER measurements were performed in HPAEC monolayers after transfection using an electrical cell substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (29).

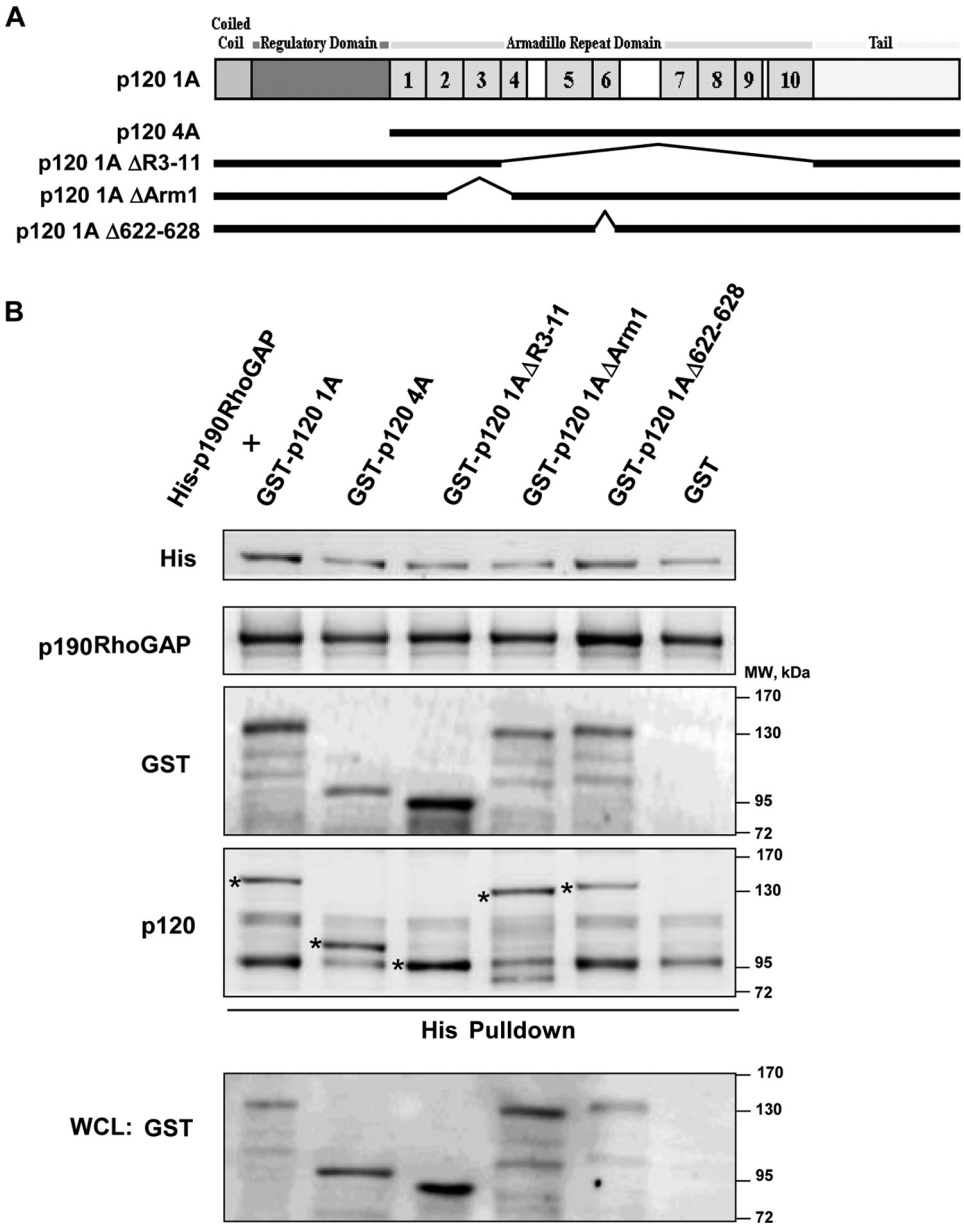

FIGURE 1. **Binding of p190RhoGAP to GST-tagged p120 catenin deletion mutants.** *A*, GST-tagged full-length p120 catenin 1A (GST-p120 1A) and related deletion mutants were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. *B*, interacting complexes were pulled down with His-tagged Dynabeads, and p120 catenin domains bound to p190RhoGAP were detected by Western blot with anti-GST and anti-p120-catenin antibodies. The *bottom panel* shows the protein content of recombinant p120-catenin and its deletion mutants in total whole cell lysates (*WCL*). *Asterisks* indicate the bands corresponding to p120–1A-catenin constructs expressed. *MW*, molecular weight.

Immunofluorescence and Image Analysis—Endothelial monolayers plated on glass coverslips were transfected with corresponding plasmids using PolyJet reagent (Signagen Laboratories, Rockville, MD). 48 hours after transfection, cells were subjected to immunofluorescence staining with GST antibody to detect transfected cells, VE-cadherin to visualize adherens junctions, and DAPI to visualize nuclei, as described previously (31, 32).

Western Blot Analysis of MYPT, MLC, Cortactin, and PAK1 Phosphorylation—Analysis of MYPT and MLC phosphorylation was used to monitor activation of Rho signaling, and levels of phosphorylated cortactin and PAK1 were assessed as readouts of Rac activation as described previously (32, 33).

Statistical Analysis—Results are expressed as mean ± S.D. of three to six independent experiments. Experimental samples were compared with controls by unpaired Student's *t* test. For multiple group comparisons, a one-way variance analysis and post hoc multiple comparisons tests were used. $p < 0.05$ was considered statistically significant.

RESULTS

Interaction between p190RhoGAP and p120-catenin—Full-length p120-catenin or deletion mutants containing an N-terminal GST tag and lacking either N terminus part, or reported Rho binding site, or clusters of Armadillo domains (Fig. 1*A*) were co-transfected in HEK 293T cells together with a His-

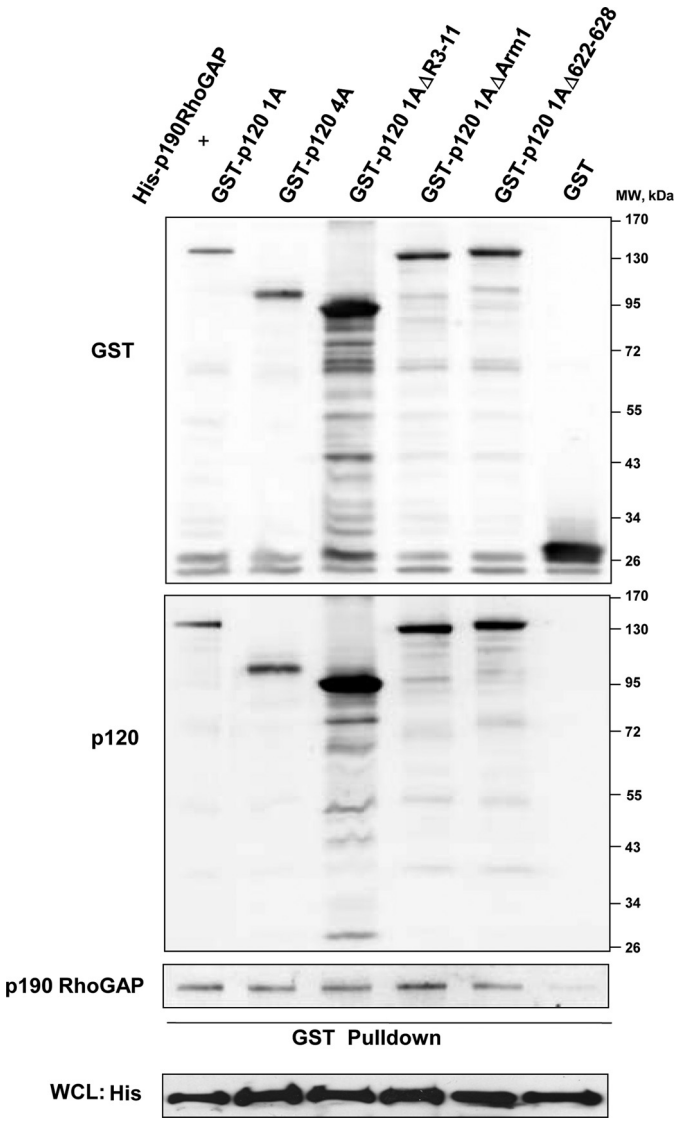

FIGURE 2. **Binding of p120 catenin truncation mutants to His-tagged p190RhoGAP.** GST-tagged full-length p120 catenin 1A (GST-p120 1A) and its deletion mutants were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. Interacting complexes were pulled down with glutathione magnetic beads, and their content was analyzed by Western blotting using antibodies to the GST tag, p120 catenin, or p190RhoGAP as indicated. The *bottom panel* depicts the protein content of recombinant His-tagged p190RhoGAP in whole cell lysates (*WCL*). *MW*, molecular weight.

tagged p190RhoGAP construct. After 48 h, clarified cell lysates were incubated either with His tag magnetic beads or glutathione magnetic beads, and the resulting complexes were analyzed by Western blotting as described under "Experimental Procedures." All p120-catenin constructs used in this analysis (*asterisk*) were detected in the His-p190RhoGAP pulldown complexes (Fig. 1*B*). The additional few bands detected by Western blotting with p120-catenin antibody are likely to correspond to the different isoforms of endogenous p120-catenin associating with p190RhoGAP. These bands were also detected when GST alone was cotransfected with p190RhoGAP.

Similar results were obtained when GST pulldown was performed (Fig. 2). p190RhoGAP was not detected when GST alone was expressed, whereas in all the other GST-p120-catenin constructs, p190RhoGAP was detected in the precipitate (Fig. 2, *bottom panel*). Similar interactions were observed when full-length p120 catenin was co-transfected with p190RhoGAP mutants lacking either the GTP-binding domain or the carboxyl (C)-terminal Rho family GTPase activating protein (GAP) domain (data not shown). Taken together, these data suggest that p190RhoGAP association with p120-catenin does not seem to involve the two main functional domains of p190RhoGAP, and the p190RhoGAP binding site is not located in the p120-catenin N terminus or central portion containing Armadillo domains. These are regions shared by all the p120-catenin constructs analyzed in this series of experiments (Fig. 1*A*).

Mapping the p120-catenin Domain Critical for p190RhoGAP-p120-catenin Association—To identify potential region involved in this interaction within the C-terminal domain of p120-catenin, we generated a series of truncated mutants by introducing stop codons at different positions within this domain (Fig. 3*A*). The mutants were generated using a PCR-based mutagenesis approach using GST-p120-catenin as a template as described under "Experimental Procedures." Once validated in terms of sequence and expression, these constructs were co-transfected with full-length p190RhoGAP into HEK 293T cells and analyzed as described above. Aside from GST alone and GST-p120 1A(1–820), all of the other GST-tagged p120-catenin mutants were found in the protein complex pulled down with His-tagged p190RhoGAP (Fig. 3*B*). These data suggest that p190RhoGAP association with p120-catenin requires the 23-amino acid stretch immediately following the last Armadillo domain of p120-catenin. We refer to this domain as CRAD (catenin-RhoGAP association domain), and we refer to the GST-p120 1A-truncated mutants as p120(1–820) and p120(1–843).

p120(1–820) Catenin Mutant Attenuates Agonist-induced EC Barrier Enhancement—The functional consequence of abrogated p120-catenin·p190RhoGAP complex formation was tested in the model of agonist-induced permeability regulation in HPAEC. Cells were transfected either with full-length p120-catenin with p120(1–820) mutant, which lacks the CRAD domain and the ability to recruit p190RhoGAP, or with the CRAD-containing truncated mutant p120(1–843). Endothelial cell response to barrier-enhancing agonist OxPAPC was evaluated by measurement of the electrical resistance across the EC monolayer. Ectopic expression of full-length p120-catenin (p120 1A) showed trend to increased TER elevation in response to OxPAPC, although this difference did not reach statistically significant levels. In turn, expression of p120(1–843) did not significantly change TER levels after OxPAPC stimulation relative to non-transfected cells. In contrast, expression of p120(1–820) mutant significantly attenuated cell response to OxPAPC (Fig. 4). Quantitative analysis of OxPAPC-induced TER changes in EC expressing p120 mutants is presented in Fig. 4*D*.

p120(1–820) Catenin Mutant Colocalizes with VE-cadherin at the Adherens Junctions and Inhibits Agonist-induced Adherens Junction Enhancement—OxPAPC-induced barrier enhancement is associated with increased assembly of adherens junction complexes and peripheral accumulation of VE-cadherin (26, 33, 34). The next experiments tested whether the lack of p190RhoGAP binding to p120 catenin would affect increased

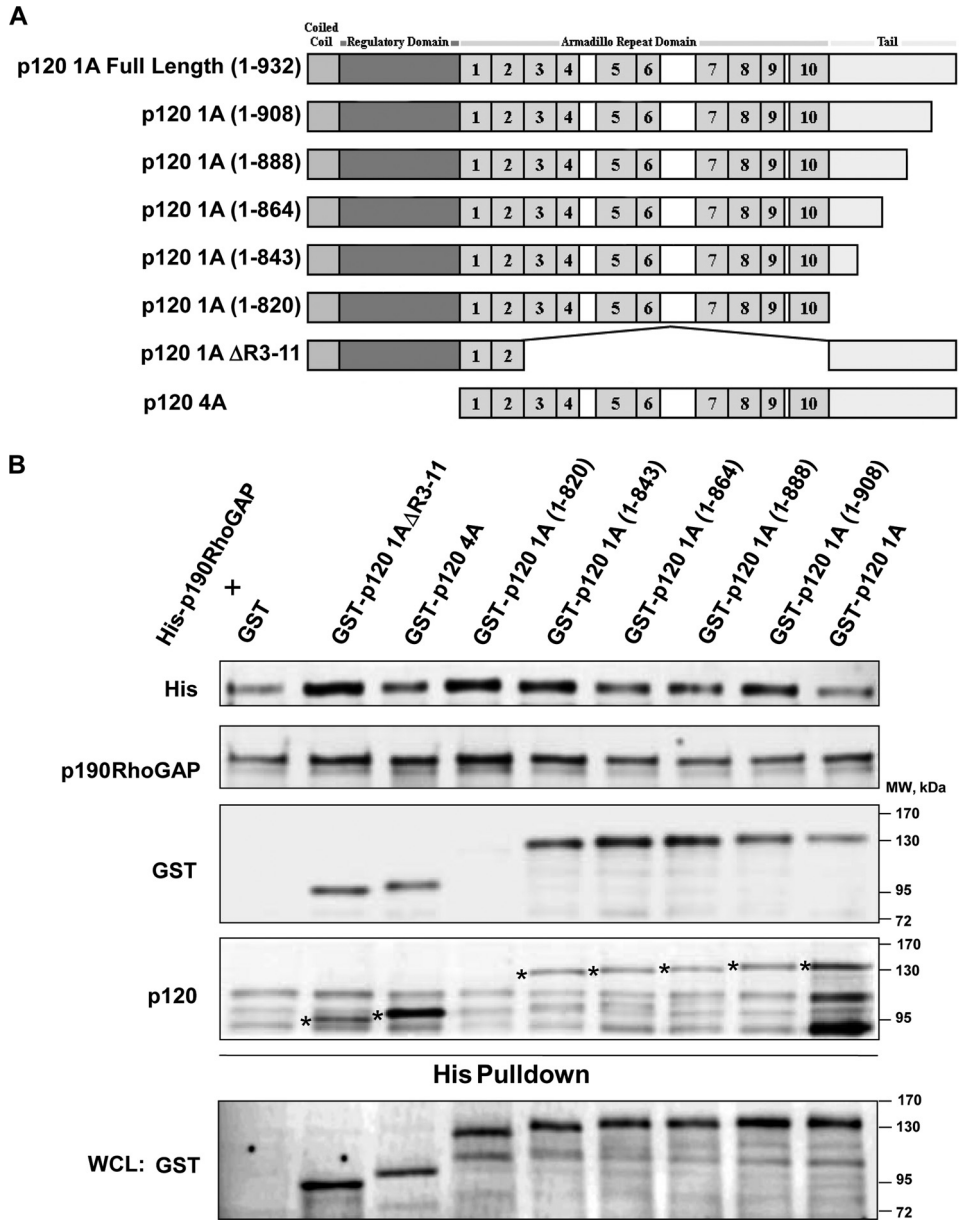

FIGURE 3. **Identification of the C-terminal polypeptide sequence in p120-catenin responsible for recruitment of p190RhoGAP.** GST-tagged full-length p120-catenin 1A (GST-p120 1A), related deletion mutants as well as truncated mutants of p120-catenin created by site specific mutagenesis (*A*) were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. Interacting complexes were pulled down with His-tagged Dynabeads (*B*), and their content was analyzed by Western blotting using antibodies to the His tag, GST tag, p120-catenin, or p190RhoGAP as indicated. *Asterisks* in *B* indicate the bands corresponding to p120-catenin constructs expressed. The *bottom panel* depicts the protein content of recombinant GST-tagged p120-catenin and its deletion mutants in whole cell lysates (*WCL*).

localization of p120 catenin and VE-cadherin to the plasma membrane caused by OxPAPC, which reflects increased adherens junction formation. Similar to endogenous proteins, ectopically expressed full-length p120-catenin (p1201A) and truncated p120(1–820) mutant co-localized with VE-cadherin at the adherens junctions of non-stimulated HPAEC (Fig. 5*A*). However, OxPAPC-induced adherens junction enhancement detected by staining of p120-catenin mutants was suppressed in cells expressing p120(1–820) (Fig. 5*B*).

p120(1–820) Catenin Attenuates OxPAPC-induced p190-RhoGAP Membrane Translocation and Modulates Rho and Rac Signaling Pathways—The translocation of p190RhoGAP to the cell membrane is facilitated by p120-catenin (25, 26). We examined whether this event is affected by expression of p120(1–820) mutant. Indeed, expression of p120(1–820) inhibited both the basal and OxPAPC-induced p190RhoGAP accumulation in the membrane fraction (Fig. 6*A*). Because the observed decrease in the pool of membrane-associated p190RhoGAP could affect local Rho signaling, we assessed the effect of the presence or the absence of CRAD on the levels of MLC phosphorylation reflecting basal Rho activation. After transfection with full-length p120 catenin, p120(1–820), or p120(1–843) constructs, HPAEC were analyzed by Western blotting using anti-pp-MLC antibody. Unlike the CRAD containing-constructs (full-length and p120(1–843)), expression of p120(1–820) lacking CRAD domain led to higher basal level of Rho

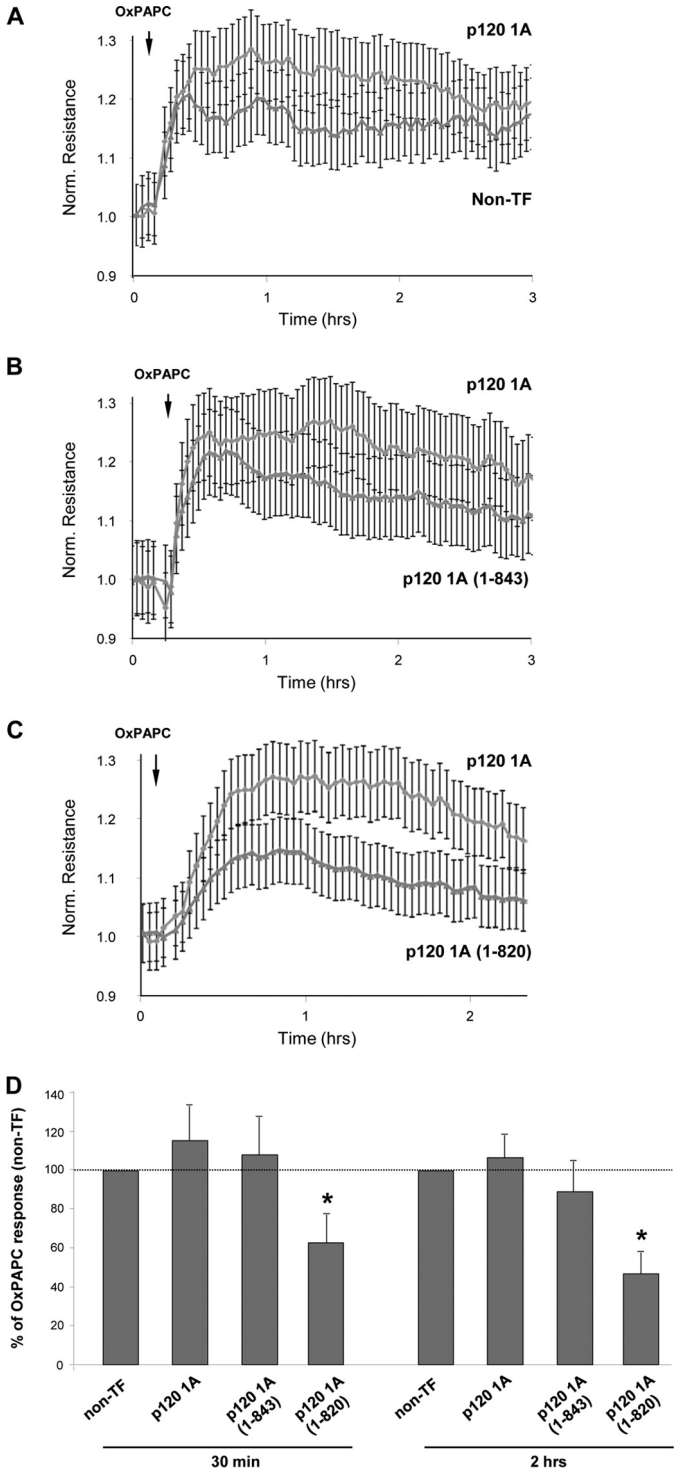

FIGURE 4. **Role of the p120-catenin CRAD domain responsible for p190RhoGAP recruitment in the OxPAPC-induced barrier enhancement.** GST-tagged full length p120 catenin (p120 1A) and truncated mutants p120(1–843) and p120(1–820) catenin were expressed in HPAEC followed by OxPAPC treatment. Time-resolved TER measurements of barrier integrity across transiently transfected HPAEC monolayers are shown in *A–C*. *Norm. Resistance*, normalized resistance; *non-TF*, non-transfected. *D*, statistical analysis of TER changes at 30 min and 2 h of OxPAPC stimulation. *, $p < 0.05$ *versus* p120 1A ($n = 5$ independent experiments).

activity and increased MLC phosphorylation mediated by Rho (Fig. 6, *B* and *C*). Increased basal Rho activation was also observed in p120(1–820) expressing HeLa cells (Fig. 6, *B* and *C*), suggesting a fundamental mechanism of Rho regulation by CRAD domain of p120-catenin.

To analyze the role of the CRAD sequence in OxPAPC-induced Rac1 activation, we transfected either full-length p120 1A, or p120(1–820), or p120(1–843) into HPAEC, and OxPAPC-induced Rac1 activation was assessed using the pull-down assay described under "Experimental Procedures." In cells transfected with p120 1A, a transient increase in the amount of GTP-loaded Rac1 is observed at 5 min. In samples where the deletion mutant lacking CRAD was used, OxPAPC failed to induce this transient increase in activated Rac1, whereas OxPAPC-induced Rac1 activation in EC expressing the mutant containing CRAD (p120(1–843)) was preserved (Fig. 6*D*). Consistent with this observation, expression of p120(1–820) catenin also suppressed activation and phosphorylation of Rac effectors PAK1 and cortactin upon OxPAPC treatment (Fig. 6*E*).

p120(1–820) Catenin Suppresses EC Barrier Recovery after Thrombin—Stimulation of endothelial cells with thrombin causes rapid and reversible Rho-dependent increase in endothelial permeability (30). In the present study, CRAD-containing, full-length p120 1A or the mutant p120(1–820) devoid of CRAD were expressed in HPAEC, and monolayer integrity was assessed by TER upon treatment with thrombin. Thrombin challenge of non-transfected HPAEC (data not shown) and cells expressing full-length p120 catenin caused a prominent but reversible permeability increase as detected by TER measurements (Fig. 7*A*). The initial phase of thrombin-mediated monolayer disruption was similar between the two constructs. However, expression of p120(1–820) markedly delayed the recovery of EC barrier after thrombin challenge (Fig. 7*A*).

Expression of p120(1–820) Catenin Mutant Prolongs Thrombin-induced RhoA Activation in HPAEC—Down-regulation of Rho signaling is an essential prerequisite of endothelial barrier recovery after agonist stimulation. We analyzed the level of Rho activation during the recovery phase following thrombin-induced monolayer disruption of endothelial cells expressing either the full-length p120 1A or p120(1–820) mutant lacking the p190RhoGAP-binding motif. Unlike p120 1A-expressing cells, cells expressing p120(1–820) exhibited delayed Rho activation, which was observed at later time points (30 and 60 min after thrombin challenge), the times normally corresponding to development of the recovery phase, Rho down-regulation and reciprocal activation of Rac signaling (Fig. 7, *A* and *B*). The recovery process can also be monitored by assessing the level of phosphorylation of downstream Rho targets, MYPT and MLC. A rapid and transient activation of RhoA-dependent signaling was evident in thrombin-stimulated HPAEC expressing full-length p120 catenin where basal levels were reestablished <30 min after thrombin treatment (Fig. 7*C*). In contrast, Rho signaling in HPAEC expressing p120(1–820) remained elevated even after 30 min of thrombin treatment (Fig. 7*C*). In turn, Rac-dependent phosphorylation of cortactin at later times after thrombin challenge, which reflects reciprocal up-regulation of Rac signaling leading to EC barrier restoration, was suppressed by expression of p120(1–820)-catenin.

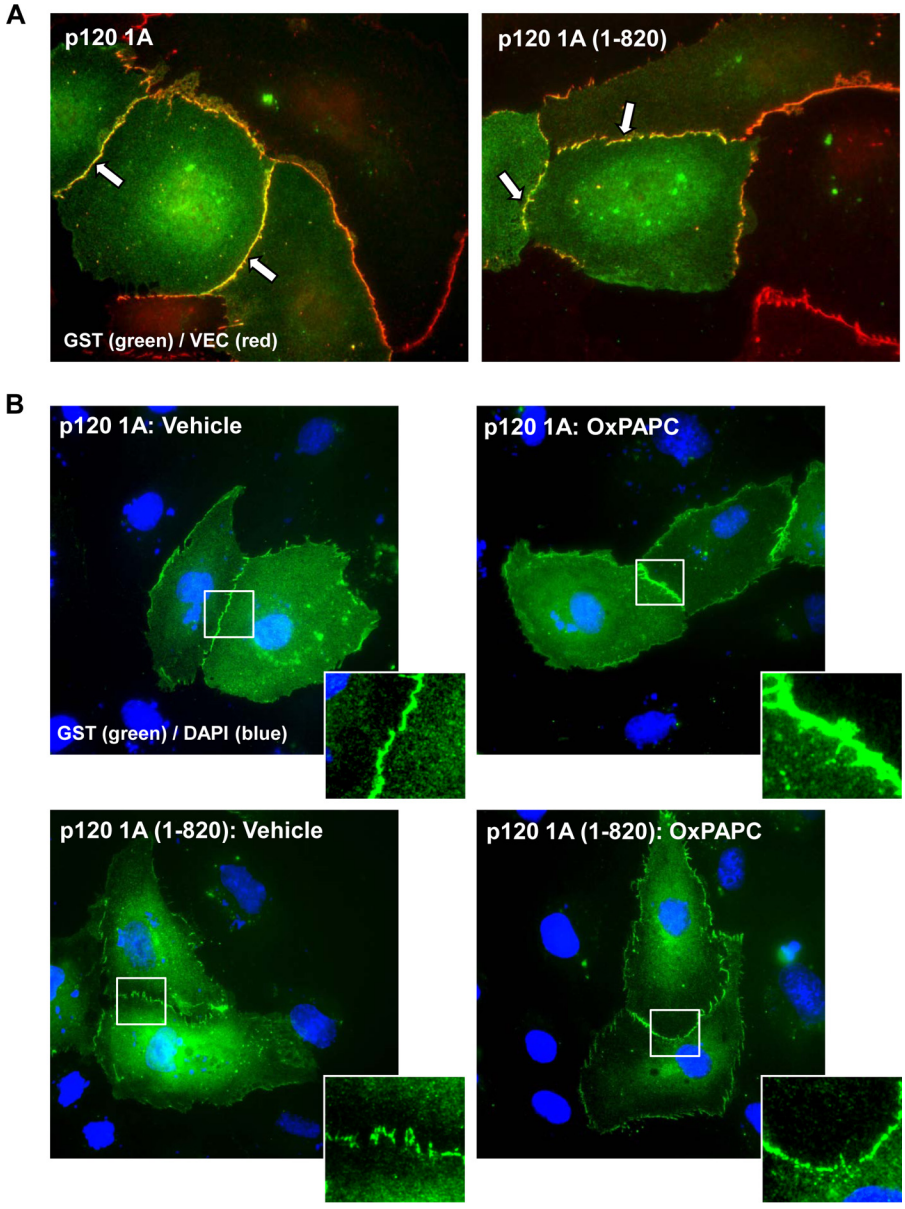

FIGURE 5. **p120(1–820) catenin mutant colocalizes with VE-cadherin to the adherens junctions but fails to accumulate at the plasma membrane upon OxPAPC treatment.** GST-tagged full-length p120 catenin (p120 1A) and truncated mutant p120(1–820) catenin were transiently transfected in HPAEC. *A*, double immunostaining with antibodies to GST tag to visualize ectopically expressed p120-catenin (*green*) and endogenous VE-cadherin (*red*) was carried out 48 h after transfection as described under "Experimental Procedures." *B*, effect of OxPAPC treatment (20 μg/ml, 30 min) on the membrane localization of p120 1A and p120(1–820) mutant. Expressed proteins were analyzed by immunofluorescence using anti-GST antibody. Cell nuclei were visualized by DAPI counterstaining.

DISCUSSION

This study identified for the first time the p120-catenin domain involved in the functional interaction with p190-RhoGAP and evaluated functional significance of uncoupled p120-catenin-p190RhoGAP interaction in the context of agonist-induced endothelial permeability regulation. Targeting of p190RhoGAP to cell junctions by p120-catenin represents an important mechanism of local regulation of Rho GTPases, which control cell-cell interactions, monolayer integrity, and vascular endothelial permeability. This study sought to identify the region within p120-catenin that is responsible for the formation of the p120-catenin·p190RhoGAP functional complex.

Published studies identified p120-catenin Armadillo domains as a region interacting with the juxtamembrane domain of cadherins (35, 36). Based on three-dimensional structure analysis, Ishiyama *et al.* (4) have shown that the bound juxtamembrane domain core peptide stretches along the N-terminal half of the p120-catenin ARM domain (ARM repeats 1–5) in the opposite orientation, suggesting that these regions are less likely to be involved in the interaction with other p120-catenin binding partners, including p190RhoGAP. We still tested involvement of N terminus and Armadillo domains in p120-catenin interaction with p190RhoGAP using N terminus and Armadillo domains p120-catenin deletion mutants. However, taking these published data into consideration, we have focused on the C-terminal tail of p120-catenin. Using a combination of deletion mutants and subsequently a series of PCR-generated truncations of the C-terminal tail of p120-catenin,

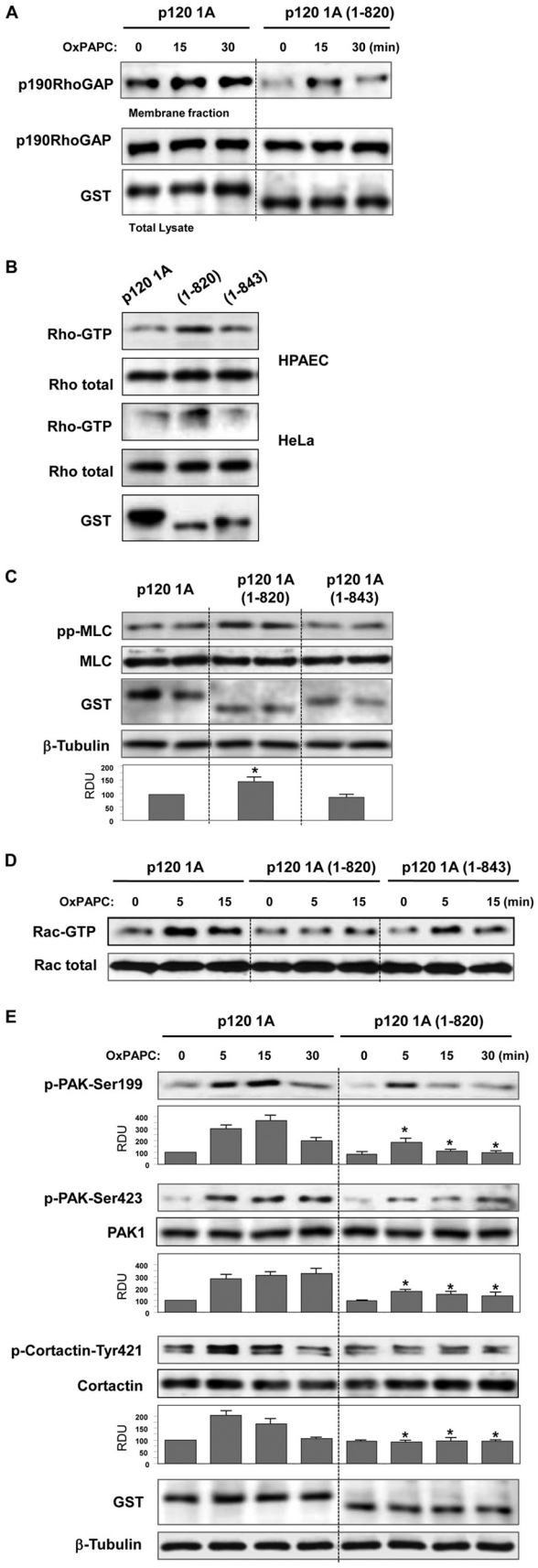

FIGURE 6. **p120(1–820) catenin attenuates OxPAPC-induced p190-RhoGAP membrane translocation and activation of Rac signaling.** GST-tagged full length p120 catenin and truncation mutants were expressed in HPAEC followed by OxPAPC treatment (15 μg/ml) for the indicated periods of time. *A*, Western blot analysis of p190RhoGAP in the membrane fractions from control and OxPAPC-treated HPAEC expressing full-length or p120(1–820)-catenin mutant (*A*, *top panel*). Total p190RhoGAP content in the cell lysates (*middle panel*) and expression levels of the recombinant p120 proteins detected by Western blot with anti-GST antibody (*bottom panel*) were used as normalization controls. *B*, Rho-GTP pulldown assay from unstimulated HPAEC and HeLa cells showing basal Rho activation (*top panel*) and total (*bottom panel*) Rho levels in cells expressing p120 1A, p120(1–820), or p120(1–843) mutants. *C*, Western blot analysis of basal phospho-MLC levels in HPAEC expressing p120 1A, p120(1–820), or p120(1–843) mutants. Samples for each condition are presented in duplicates. Western blot detection of GST tag (*middle panel*) was used to monitor recombinant p120-catenin expression; detection of β-tubulin (*bottom panel*) was used as normalization control. *RDU*, relative density units. *D*, Rac-GTP pulldown assay showing active Rac (*top panel*) and total (*bottom panel*) Rac levels. *E*, Western blot analysis of phospho-PAK1 and phospho-cortactin levels in OxPAPC-treated HPAEC expressing full-length and p120(1–820)-catenin mutant using phospho-site-specific PAK1 and cortactin antibodies. GST and β-tubulin staining were included to account for construct expression levels and sample loading, respectively. Bar graphs represent quantitative densitometry analysis of Western blot data. *, $p < 0.05$ *versus* p120 1A ($n = 5$ independent experiments).

we have identified a stretch of 23 amino acids (amino acids 821–843) following Armadillo domain-10, designated as the CRAD domain, which mediates the formation of functional p120-catenin·p190RhoGAP complex. We further performed analysis of the CRAD domain homology with other binding proteins. Results of BLAST search for homologies were negative and did not yield any positive hits, suggesting that this is a unique sequence rather than consensus sequence shared by other p190RhoGAP interactors. It is also important to note that, although the role of CRAD domain in p120-catenin·p190RhoGAP association was tested extensively in the forward and reverse pulldown assays of p190RhoGAP and p120-catenin truncation mutants co-expressed in mammalian cell system, we cannot exclude the presence of intermediate linker protein involved in these interactions.

Control of Rho signaling by p190RhoGAP is critical for dynamic EC barrier regulation. We have previously discovered that stimulation of pulmonary endothelium with OxPAPC promoted p120-catenin/p190RhoGAP association, tyrosine phosphorylation, and recruitment of p190RhoGAP to adherens junctions, leading to enhancement of the endothelial barrier and reciprocal reduction of Rho activity and elevation of Rac signaling via Rac-Rho cross-talk mechanisms (26). Important role of p190RhoGAP activity in control of vascular endothelial barrier was demonstrated in experiments with molecular inhibition of p190RhoGAP, which exacerbated Rho-dependent endothelial permeability *in vitro* and lung vascular leak in the animal models of ventilator-induced lung injury (26).

Uncoupling of p120-catenin·p190RhoGAP association achieved in the present study by expression of the p120-catenin mutant lacking the p190RhoGAP binding site had important functional implications. Expression of the p120 1A(1–820) mutant lacking the CRAD domain suppressed the OxPAPC-induced p190RhoGAP translocation to cell membrane compartment, caused an increase in basal Rho signaling and reciprocal decrease in basal Rac activation in cells expressing the p120 1A(1–820) mutant. As a result, expression of truncated mutant lacking the CRAD domain (p120 1A(1–820)) attenuated endothelial barrier enhancing response to OxPAPC. In contrast, expression of the full-length construct or the truncation mutant containing the CRAD domain (p120 1A(1–843))

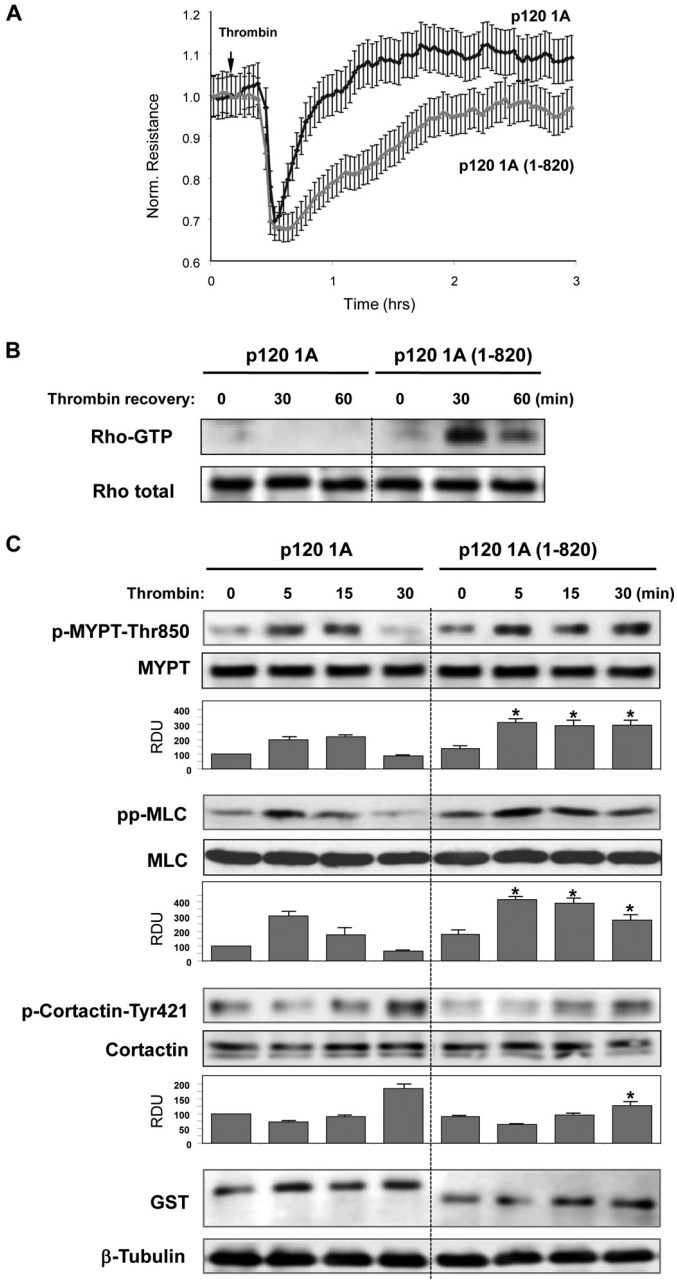

FIGURE 7. **p120(1–820) catenin delays thrombin-induced barrier restoration and prolongs thrombin-induced activation of Rho signaling.** GST-tagged full-length p120 catenin (p120 1A) and its truncation mutant p120(1–820) were expressed in HPAEC followed by thrombin treatment (0.2 units/ml). *A*, TER measurements showing thrombin-induced permeability and barrier restoration in HPAEC monolayers. Thrombin-induced barrier dysfunction was markedly prolonged by expression of p120(1–820) catenin. *Norm. Resistance*, normalized resistance. *B*, Rho-GTP pulldown assay on thrombin-treated HPAEC expressing full-length or p120(1–820) catenin mutant during the recovery phase. *C*, Western blot analysis of phospho-MYPT, phospho-MLC, and phospho-cortactin levels in HPAEC expressing full-length and p120(1–820) catenin mutant upon thrombin stimulation for the indicated periods of time. GST and β-tubulin staining were included in *C* to account for construct expression levels and sample loading, respectively.

did not significantly alter the OxPAPC-induced endothelial barrier enhancement when compared with non-transfected cells. Under conditions of thrombin-induced, Rho-mediated increase in EC permeability, expression of p120(1–820) led to a sustained activation of Rho and its downstream signaling after thrombin challenge and delayed activation of Rac signaling. As a result, EC monolayers expressing the truncated mutant lacking CRAD exhibited a lower ability to recover from the disruptive effect of thrombin. Taken together, these data suggest that p120-catenin coordinates the activities of Rho and Rac pathways in response to OxPAPC and that the C terminus domain of p120-catenin containing amino acids 821–843 is important for this switch mechanism. Whether all observed effects can be attributable to the CRAD fragment itself or to other portions of the C terminus domain of p120-catenin is yet to be clarified.

Previous reports described p120-catenin functional interactions with the small GTPases Rho, Rac, and Cdc42 (18, 24, 25, 37, 38). Among these three GTPases, only direct interaction with RhoA was reported (22, 24). Such an interaction, mediated through the p120-catenin Armadillo domain and inhibited by cadherin binding, inhibited RhoA activity, which led the authors to propose a mechanism whereby p120-catenin would play a role of a "recruiting agent" for RhoA to nascent cell contacts. Further evidence has shown that p120-catenin constitutes a hub regulating small GTPase activities as exemplified by its interaction with regulators of Rac and Rho GTPases as the guanine nucleotide exchange factor Vav2 (24) and p190RhoGAP (25, 26, 39).

Interestingly, our current study shows additional p120-catenin associated inhibitory effect toward Rho via p190-RhoGAP binding to p120-catenin amino acids 820–843 domain in the C-terminal tail located immediately downstream of the last Armadillo domain-10. Incidentally, this region was recently shown to form two α helices that fold over the hydrophobic surface of Armadillo domain R9 (4) Taken together, these observations suggest the existence of a regulatory mechanism mediated by intramolecular folding of p120-catenin in the recruitment of p190RhoGAP and ultimately in the local inhibition of Rho activity. These conformation changes are apparently induced by cell stimulation with barrier-enhancing agonists. However, the exact events involved in such a mechanism are yet to be identified but do reveal a potential regulatory role of the C-terminal tail of p120-catenin.

In conclusion, our results suggest a key role for p120-catenin C-terminal CRAD domain (amino acids 821–843) in p190RhoGAP subcellular targeting, which is essential for local regulation of agonist-induced Rho and Rac activities and dynamic control of endothelial barrier under normal conditions and during vascular barrier dysfunction associated with acute lung injury, pulmonary and brain edema, atherosclerosis, and other pathological conditions.

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